



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(S1) International Patent Classification <sup>6</sup> : <b>A61K 31/00, 31/135, 31/22, 31/34, 31/35, 31/38, 31/41, 31/42, 31/425, 31/44, 31/53, 31/55</b>		<b>A3</b>	(11) International Publication Number: <b>WO 96/40113</b>  (43) International Publication Date: 19 December 1996 (19.12.96)
(21) International Application Number: <b>PCT/US96/09960</b>		(81) Designated States: AL, AM, AU, AZ, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IL, IS, JP, KG, KP, KR, KZ, LK, LR, LS, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TJ, TM, TR, TT, UA, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 7 June 1996 (07.06.96)			
(30) Priority Data: <b>476,137 7 June 1995 (07.06.95) US</b>			
(71) Applicant: SUGEN, INC. [US/US]; 515 Galveston Drive, Redwood City, CA 94063 (US).		Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
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(74) Agents: CORUZZI, Laura, A. et al.; Pennie & Edmonds, 1155 Avenue of the Americas, New York, NY 10036 (US).			

(54) Title: PHOSPHATASE INHIBITORS

## (57) Abstract

The present invention relates to organic molecules capable of inhibiting protein tyrosine phosphatase activity. The invention further relates to the use of such molecules to modulate or regulate signal transduction by inhibiting protein tyrosine phosphatase activity. Finally, the invention relates to the use of such molecules to treat various disease states including diabetes mellitus.

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GA	Gabon			VN	Viet Nam

## INTERNATIONAL SEARCH REPORT

International Application No  
PL1/US 96/09960

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/00 A61K31/135 A61K31/22 A61K31/34 A61K31/35
A61K31/38 A61K31/41 A61K31/42 A61K31/425 A61K31/44
A61K31/53 A61K31/55

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ANN. REV. CELL BIOL., vol. 8, 1992, pages 429-462. XP000609794 S.S. TAYLOR ET AL.: "Structural framework for the protein kinase family." ---	
A	US,A,5 198 333 (DEWANCKELE ET AL.) 30 March 1993 cited in the application ---	
A	US,A,3 870 725 (HUGHES ET AL.) 11 March 1975 cited in the application ---	
A	US,A,3 850 939 (ELSLAGER ET AL.) 26 November 1974 cited in the application -----	

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

Date of the actual completion of the international search

25 November 1996

Date of mailing of the international search report

05.12.96

## Name and mailing address of the ISA

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## **INTERNATIONAL SEARCH REPORT**

**International application No.**

PCT/LJ 96/09960

**Box I Observations where certain claims were found unsearchable (Continuation of item I of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
  
  2.  Claims Nos.: 1-7 because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
  
See next page.
  
  3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### **Remark on Protest**

- The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 96/09960

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

In view of the large number of compounds, which are defined by the general definitions used in claims 1-7, the search had to be restricted for economic reasons. The search was limited to the compounds for which pharmacological data was given and/or the compounds mentioned in the claims, and to the general idea underlying the application.

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/09960

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A-5198333	30-03-93	EP-A-	0448841	02-10-91
		DE-T-	69001809	05-01-94
		JP-A-	4221950	12-08-92
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		CH-A-	545812	15-02-74
		DE-A-	2213558	05-10-72
		FR-A-	2132133	17-11-72
US-A-3850939	26-11-74	AT-B-	332402	27-09-76
		AU-B-	473343	17-06-76
		AU-A-	6575974	21-08-75
		BE-A-	811267	17-06-74
		CH-A-	579079	31-08-76
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		FR-A-	2218103	13-09-74
		GB-A-	1420570	07-01-76
		JP-A-	49110671	22-10-74
		NL-A-	7402268	22-08-74

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**PHOSPHATASE INHIBITORS****1. INTRODUCTION**

The present invention relates to compounds capable of modulating and/or regulating the activity of phosphotyrosine phosphatases that regulate signal transduction. In particular, the present invention relates to the use of such compounds for the treatment of diseases caused by dysfunctional signal transduction. This application is a continuation-in-part of U.S. Application Serial No. 08/476,137, filed June 07, 1995.

**2. BACKGROUND OF THE INVENTION****15 2.1. Signal Transduction**

Cellular signal transduction is a fundamental mechanism whereby external stimuli that regulate diverse cellular processes are relayed to the interior of cells. The biochemical pathways through which signals are relayed within cells comprise a circuitry of directly or functionally connected interactive proteins. One of the key biochemical mechanisms of signal transduction involves the reversible phosphorylation of tyrosine residues on proteins. The phosphorylation state of a protein may affect its conformation and/or enzymic activity as well as its cellular location. The phosphorylation state of a protein is modified through the reciprocal actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) at various specific tyrosine residues.

30

**2.2. Protein Tyrosine Kinases And Phosphatases**

A common mechanism by which receptors regulate cell function is through an inducible tyrosine kinase activity which is either endogenous to the receptor or is imparted by other proteins that become associated with the receptor. (Darnell et al., 1994, Science 264:1415-1421; Heldin, 1995, Cell 80:213-223; Pawson, 1995, Nature 373:573-580).

Protein tyrosine kinases comprise a large family of transmembrane receptor and intracellular enzymes with multiple functional domains (Taylor et al., 1992 Ann. Rev. Cell Biol. 8:429-62). The binding of ligand allosterically 5 transduces a signal across the cell membrane where the cytoplasmic portion of the PTKs initiates a cascade of molecular interactions that disseminate the signal throughout the cell and into the nucleus. Many receptor protein tyrosine kinase (RPTKs), such as epidermal growth factor 10 receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) undergo oligomerization upon ligand binding, and the receptors self-phosphorylate (via autophosphorylation or transphosphorylation) on specific tyrosine residues in the cytoplasmic portions of the receptor (Schlessinger and 15 Ullrich, 1992, Neuron, 9:383-91, Heldin, 1995, Cell 80:213-223). Cytoplasmic protein tyrosine kinases (CPTKs), such as Janus kinases (e.g., JAK1, JAK2, TYK2), Src kinases (e.g., src, lck, fyn) are associated with receptors for cytokines (e.g., IL-2, IL-3, IL-6, erythropoietin) and interferons, and 20 antigen receptors. These receptors also undergo oligomerization, and have tyrosine residues that become phosphorylated during activation, but the receptor polypeptides themselves do not possess kinase activity.

Like the PTKs, the protein tyrosine phosphatases (PTPs) 25 comprise a family of transmembrane and cytoplasmic enzymes, possessing at least an approximately 230 amino acid catalytic domain containing a highly conserved active site with the consensus motif [I/V]HCXAGXXR[S/T]G. The substrates of PTPs may be PTKs which possess phosphotyrosine residues or the 30 substrates of PTKs. (Hunter, 1989, Cell 58:1013-16; Fischer et al., 1991, Science 253:401-6; Saito & Streuli, 1991, Cell Growth and Differentiation 2:59-65; Pot and Dixon, 1992, Biochem. Biophys. Acta, 1136:35-43).

Transmembrane or receptor-like PTPs (RPTPs) possess an 35 extracellular domain, a single transmembrane domain, and one or two catalytic domains followed by a short cytoplasmic tail. The extracellular domains of these RPTPs are highly

divergent, with small glycosylated segments (e.g., RPTP $\alpha$ , RPTP $\epsilon$ ), tandem repeats of immunoglobulin-like and/or fibronectin type III domains (e.g., LAR) or carbonic anhydrase like domains (e.g., RPTP $\gamma$ , RPTP $\beta$ ). These 5 extracellular features might suggest that these RPTPs function as a receptor on the cell surface, and their enzymatic activity might be modulated by ligands. Intracellular or cytoplasmic PTPs (CPTPs), such as PTP1C, PTP1D, typically contain a single catalytic domain flanked by 10 several types of modular conserved domains. For example, PTP1C, a hemopoietic cell CPTP is characterized by two Src-homology 2 (SH2) domains that recognize short peptide motifs bearing phosphotyrosine (pTyr).

In general, these modular conserved domains influence 15 the intracellular localization of the protein. SH2-containing proteins are able to bind pTyr sites in activated receptors and cytoplasmic phosphoproteins. Another conserved domain known as SH3 binds to proteins with proline-rich regions. A third type known as pleckstrin-homology (PH) 20 domain has also been identified. These modular domains have been found in both CPTPs and CPTPs as well as in non-catalytic adapter molecules, such as Grbs (Growth factor Receptor Bound), which mediate protein-protein interactions between components of the signal transduction pathway 25 (Skolnik et al., 1991, Cell 65:83-90; Pawson, 1995, Nature 373:573-580).

Multiprotein signaling complexes comprising receptor subunits, kinases, phosphatases and adapter molecules are assembled in subcellular compartments through the specific 30 and dynamic interactions between these domains with their binding motifs. Such signaling complexes integrate the extracellular signal from the ligand-bound receptor and relay the signal to other downstream signaling proteins or complexes in other locations inside the cell or in the 35 nucleus (Koch et al., 1991, Science 252:668-674; Pawson, 1994, Nature 373:573-580; Mauro et al., 1994, Trends Biochem Sci 19:151-155; Cohen et al., 1995, Cell 80:237-248).

**2.3. Abnormal Signal Transduction In Human Diseases**

The levels of tyrosine phosphorylation required for normal cell growth and differentiation at any time are achieved through the coordinated action of PTKs and PTPs.

5 Depending on the cellular context, these two types of enzymes may either antagonize or cooperate with each other during signal transduction. An imbalance between these enzymes may impair normal cell functions leading to metabolic disorders and cellular transformation.

10 For example, insulin binding to the insulin receptor, which is a PTK, triggers a variety of metabolic and growth promoting effects such as glucose transport, biosynthesis of glycogen and fats, DNA synthesis, cell division and differentiation. Diabetes mellitus which is characterized by 15 insufficient or a lack of insulin signal transduction can be caused by any abnormality at any step along the insulin signaling pathway. (Olefsky, 1988, in "Cecil Textbook of Medicine," 18th Ed., 2:1360-81).

It is also well known, for example, that the 20 overexpression of PTKs, such as HER2, can play a decisive role in the development of cancer (Slamon et al., 1987, Science 235:77-82) and that antibodies capable of blocking the activity of this enzyme can abrogate tumor growth (Drebin et al., 1988, Oncogene 2:387-394). Blocking the signal 25 transduction capability of tyrosine kinases such as Flk-1 and the PDGF receptor have been shown to block tumor growth in animal models (Millauer et al., 1994, Nature 367:577; Ueno et al., Science, 252:844-848).

Relatively less is known with respect to the direct role 30 of tyrosine phosphatases in signal transduction; PTPs may play a role in human diseases. For example, ectopic expression of RPTP $\alpha$  produces a transformed phenotype in embryonic fibroblasts (Zheng et al., Nature 359:336-339), and overexpression of RPTP $\alpha$  in embryonal carcinoma cells causes 35 the cells to differentiate into a cell type with neuronal phenotype (den Hertog et al., EMBO J 12:3789-3798). The gene for human RPTP $\gamma$  has been localized to chromosome 3p21 which

is a segment frequently altered in renal and small lung carcinoma. Mutations may occur in the extracellular segment of RPTPy which renders a RPTP that no longer respond to external signals (LaForgia et al., Wary et al., 1993, Cancer Res 52:478-482). Mutations in the gene encoding PTP1C (also known as HCP, SHP) are the cause of the *motheaten* phenotype in mice which suffer severe immunodeficiency, and systemic autoimmune disease accompanied by hyperproliferation of macrophages (Schultz et al., 1993, Cell 73:1445-1454). PTP1D 10 (also known as Syp or PTP2C) has been shown to bind through SH2 domains to sites of phosphorylation in PDGFR, EGFR and insulin receptor substrate 1 (IRS-1). Reducing the activity of PTP1D by microinjection of anti-PTP1D antibody has been shown to block insulin or EGF-induced mitogenesis (Xiao et 15 al., 1994, J Biol Chem 269:21244-21248).

It has been reported that some of the biological effects of insulin can be mimicked by vanadium salts such as vanadates and pervanadates. Vanadates and pervanadates are known to be non-specific phosphatase inhibitors. However, 20 this class of compounds is toxic because each compound contains a heavy metal (U.S. Patent No. 5,155,031; Fantus et al., 1989, Biochem., 28:8864-71; Swarup et al., 1982, Biochem. Biophys. Res. Commun. 107:1104-9).

25

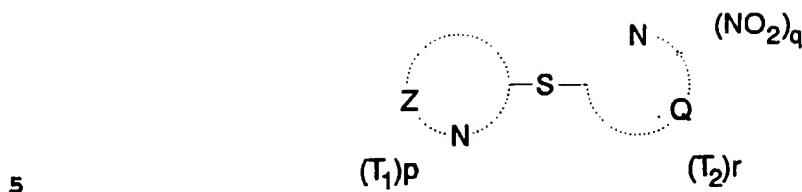
### 3. SUMMARY OF THE INVENTION

The present invention is directed to compounds capable of modulating and/or regulating receptor-mediated signal transduction by inhibiting the activity of protein tyrosine phosphatases (PTPs). The compounds of the invention can 30 inhibit the dephosphorylation of phosphotyrosine residues of a substrate wherein the substrate relays signals in a signaling pathway. The compounds may also functionally mimic the effect on a signal transduction of ligand binding to a signaling receptor. The invention is also directed to 35 methods of inhibiting protein tyrosine phosphatase activity by contacting cells with an effective amount of a compound of the present invention or a pharmaceutically acceptable salt

thereof. Further, the invention encompasses methods of treating disease states in mammals, including humans, which are ameliorated by modulating and/or regulating signal transduction through the inhibition of protein tyrosine phosphatase activity. Such disease states or disorders include but are not limited to diabetes, cancer, immune and neurological disorders.

The compounds of the present invention inhibit phosphatase activity in cells, so that dephosphorylation of various tyrosine kinases, or other phosphatase substrates involved in the signaling pathway is decreased. As a result, the appearance of phosphotyrosine residues on such substrates will increase. A number of different types of compounds, which may or may not share certain structural features are encompassed within the invention. Although not limited to any theory or explanation, the compounds of the invention are thought to demonstrate such activity by mimicking the transition state of phosphates departing phosphotyrosines. Two features believed to be involved in conferring such activities are (a) an aryl group that mimics the structure of tyrosine and/or tyrosine backbone, and (b) a highly diffuse negatively charged moiety that mimics the negatively charged phosphate binding of divalent metal ions, such as Mg<sup>++</sup>, Mn<sup>++</sup>, Zn<sup>++</sup> and the like. Preferably, the negatively charged moiety is located sufficiently close to the aryl group so that the negative charge could diffuse into the aryl moiety. Functional groups which contribute to the negatively charged moiety include, but are not limited to hydroxy-ketone and acidic groups; examples of such groups are shown in Formula IV, R<sub>2</sub> and R<sub>3</sub>, and in Formula VII, R<sub>6</sub>, infra. An aryl moiety that mimics tyrosine is exemplified by the substituted five membered ring shown in Formula III infra.

For example, the compounds of the present invention are heterocyclic nitrogen containing compounds of formula I:



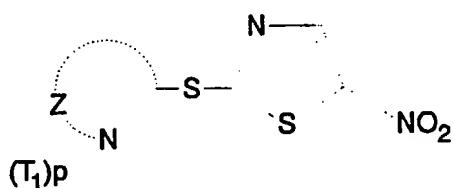
Formula I

wherein:

- 10 each of Z and Q, which may be the same or different, represents the atoms necessary to complete an unsubstituted or substituted nitrogen containing heterocyclic ring;
- each of T<sub>1</sub> and T<sub>2</sub> which may be the same or different represents alkyl, substituted alkyl, cycloalkyl, substituted 15 cycloalkyl, aryl, substituted aryl, aryloxy, halogen, cyano, hydroxy, carboxyl, sulfo, carbamoyl, acyl, acylamino, thiacylamino, sulfamoyl, or sulfonamido; q = 1, 2, or 3, and p and r = 0, 1 or 2. In formula I, the nitrogen containing heterocyclic nucleus identified by the term "Q" is preferably 20 nitropyridine or nitrothiazole. In addition, the present invention encompasses pharmaceutically acceptable salts or analogs of the above compounds.

Preferred compounds of the present invention include those of formula II:

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30

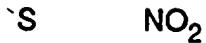
Formula II

wherein Z, T<sub>1</sub> and p are as defined above.

- In another embodiment of the present invention, the 35 compounds of the present invention are described by the formula III:

N—

A—S—



5

## Formula III

wherein A represents (i) a substituted or unsubstituted monocyclic five or six membered ring having 1-4 hetero ring atoms, at least one of which is nitrogen, the remainder of which are selected from nitrogen, oxygen or sulfur, e.g., pyridine, pyrrole, imidazole, thiazole, isothiazole, isoxazole, furazan, pyrrolidine, piperidine, imidazolidine, piperazine, oxazole, tetrazole, pyrazole, triazole, oxadiazole, thiodiazole; (ii) a substituted or unsubstituted monocyclic or fused bicyclic six to ten membered ring having 1 to 4 hetero ring atoms, one of which is nitrogen and the remainder of which are nitrogen, oxygen or sulfur, e.g., indole, quinoxaline, quinazoline, quinoline, isoquinoline, purine; or (iii) a substituted or unsubstituted monocyclic or fused polycyclic saturated or unsaturated ring having three to 15 atoms, which are carbon, sulfur, nitrogen or oxygen.

The heterocyclic rings defined above may be saturated or unsaturated. The unsaturated rings or heteroaromatic group may, if desired, bear one or more substituents which do not substantially adversely affect the activity of the compound of formula I. Exemplary of such substituents are alkyl, alkoxy, phenoxy, alkenyl, alkynyl, phenylalkyl, hydroxyalkyl, haloalkyl, aryl, arylalkyl, alkyloxy, alkylthio, alkenylthio, phenylalkylthio, hydroxyalkyl-thio, alkylthiocarbamylthio, phenyl, cyclohexyl, pyridyl, piperidinyl, alkylamino, amino, nitro, mercapto, cyano, hydroxyl, a halogen atom, an oxygen atom (forming a ketone or N-oxide) or a sulphur atom (forming a thione).

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### 3.1. Definitions

By the term "alkyl" as used herein is meant a straight or branched chain saturated hydrocarbon group having from 1 to 20 carbons such as methyl, ethyl, isopropyl, n-butyl, s-5 butyl, t-butyl, n-amyl, isoamyl, n-hexyl, n-octyl and n-decyl. The terms "alkenyl" and "alkynyl" are used to mean straight or branched chain hydrocarbon groups having from 2 to 10 carbons and unsaturated by a double or triple bond respectively, such as vinyl, allyl, propargyl, 1-methylvinyl, 10 but-1-enyl, but-2-enyl, but-2-ynyl, 1-methylbut-2-enyl, pent-1-enyl, pent-3-enyl, 3-methylbut-1-ynyl, 1,1-dimethylallyl, hex-2-enyl and 1-methyl-1-ethylallyl. The term "phenylalkyl" means the aforementioned alkyl groups substituted by a phenyl group such as benzyl, phenethyl, phenopropyl, 1-benzylethyl, 15 phenobutyl and 2-benzylpropyl. The term "aryl" as used herein is meant to include a monocyclic, bicyclic, tricyclic or other polycyclic compounds, wherein at least one ring is aromatic including aromatic hydrocarbons or hetero-aromatic hydrocarbons having heteroaromatic atoms such as nitrogen, 20 sulfur and oxygen. The term "hydroxy-alkyl" means the aforementioned alkyl groups substituted by a single hydroxyl group such as 2-hydroxyethyl, 2-hydroxypropyl, 3-hydroxypropyl, 4-hydroxybutyl, 1-hydroxybutyl and 6-hydroxyhexyl. The terms "alkylthio, alkenylthio, 25 alkynylthio, alkylthio, hydroxy-alkylthio and phenyl-alkylthio" as used herein mean the aforementioned alkyl, alkenyl, alkynyl, hydroxy-alkyl and phenyl-alkyl groups linked through a sulfur atom to group R.

The term "substituted" as used herein means that the 30 group in question, e.g., alkyl group, aryl group, etc., may bear one or more substituents including but not limited to halogen, hydroxy, cyano amino, nitro, mercapto, carboxy and other substituents known to those skilled in the art.

The terms "saturated" as used herein means an organic 35 compound with neither double or triple bonds. The term "unsaturated" as used herein means an organic compound containing either double or triple bonds.

**4. BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1. Dose response effect of compound 10 on the level of phosphotyrosine (pTyr) residues on insulin receptor over time.

- 5 Figure 2. Dose response effect of 2-methyl-5,6,8-trihydroxy-9-(5,6,8-trihydroxy-2-methyl-4H-naphtho[2,3,b]pyrano-4-one-9-yl)4H-naphtho[2,3,b]pyran-4-one on the uptake of 2-deoxy-<sup>3</sup>H-glucose in 3T3-L1 cells, a preadipocyte cell line.

10

**5. DETAILED DESCRIPTION OF THE INVENTION**

The present invention is directed to compounds capable of inhibiting the activity of protein tyrosine phosphatases (PTPs) for modulating or triggering signal transduction.

- 15 The compounds of the invention can inhibit the dephosphorylation of phosphotyrosine residues of a substrate wherein the substrate relays signals in a signaling pathway. The compounds may also functionally mimic the effect on a signal transduction of ligand binding to a signaling receptor. The invention is further directed to the use of such compounds in modulating or triggering signal transduction, and in the regulation of cellular processes that are controlled by signal transduction through the inhibition of the activity of PTPs by the compounds. The 25 invention further provides for the use of such compounds in the treatment of a subject having a disorder caused by dysfunctional signal transduction.

In one embodiment of the invention, the compounds of the invention are capable of inhibiting the activity of protein 30 tyrosine phosphatases, that are transmembrane or intracellular, and that may have one or more characteristic catalytic domains. The amino acid sequences of the PTPs in the catalytic domains may include but are not limited to [I/V]HCXAGXXR[S/T]G (single-letter amino acid code; X is any 35 amino acid). In addition, the PTPs may possess one or more modular conserved domains, which include but are not limited to, SH2, SH3 and PH domains. In a specific embodiment of the

invention, the compounds of the invention can be used to inhibit the phosphatase activity of PTP1B (Charbonneau et al., 1989, Proc. Natl Acad Sci USA, 86: 5252-5256), T-cell PTP (Cool et al., 1989, Proc Natl Acad Sci USA, 86: 5257-5261, PTP1C (Shen et al., 1991, Nature, 352: 736-739), PTP1D (Vogel et al., 1993, Science 259: 1611-1614), RPTP $\alpha$ , RPTP $\beta$ , RPTP $\gamma$  (Kaplan et al., 1990, Proc Natl Acad Sci USA, 87: 7000-7004), RPTP $\sigma$  (Yan et al., 1993, J Biol Chem 268: 24880-24886), RPTP $\kappa$  (Jiang et al., 1993, Mol Cell Biol, 13: 2942-2951) and CD45 (Charbonneau et al., 1988, Proc Natl Acad Sci USA 85: 7182-7186). The PTPs preferred in the invention are of human origin. Inhibition of phosphatase activity that is substantially specific to a PTP or a set of PTPs in a signaling pathway is preferred.

The term "signal transduction" as used herein is not limited to transmembrane signaling, and includes the multiple pathways that branch off throughout the cell and into the nucleus. Such signaling pathways may include but are not limited to the Ras pathway (Schlessinger, 1994, Curr Opin Genet Dev 4:25-30), the JAK/STAT pathways (Sadowski et al., 1994, Science 261:1739-1744), the phosphoinositide 3-kinase pathway and the phospholipase C- $\gamma$  pathway. As used herein, the term "modulation" or "modulating" shall mean upregulation or downregulation of a signaling pathway. Cellular processes under the control of signal transduction may include, but are not limited to, transcription of specific genes; normal cellular functions, such as metabolism, proliferation, differentiation, adhesion, apoptosis and survival; as well as abnormal processes, such as transformation, blocking of differentiation and metastasis.

A signal may be triggered by the binding of a ligand to its receptor on the cell surface, and the signal is relayed and propagated by the phosphorylation or dephosphorylation of specific tyrosine residues on various substrates inside the cell. The specific interactions between the PTKs, PTPs and their substrates may involve the formation of a transient or stable multimolecular complex on the inner face of the plasma

membrane or in other subcellular compartments including the nucleus. A substrate may contain one or more tyrosine residues that are specifically phosphorylated or dephosphorylated by PTKs or PTPs in the signaling pathway.

5 Such substrates may include the receptor and its subunits, molecules associated with or recruited to the receptor such as cytoplasmic kinases, cytoplasmic phosphatases, adapter molecules, cytoskeletal proteins and transcription factors.

The term receptor as used herein may include, but is not limited to, insulin receptor, members of the insulin-like growth factor receptor family, epidermal growth factor receptor family, fibroblast growth factor receptor family, hepatocyte growth factor receptor family, vascular endothelial growth factor receptor family, neurotrophin 15 receptor (trk) family, the T-cell receptor, the B cell receptor and members of the Type I-IV cytokine receptor families (Heldin, 1995, Cell. 80: 213-223; Taniguchi, 1995, Science, 268: 251-255). Adapter molecules that are substrates may include the Grb proteins, IRS-1, Zap-70 and 20 Shc (Pawson et al., 1995, Nature 373: 573-580). Cytoskeletal proteins such as actin and transcription factors such as the STAT proteins (Ihle et al., Trends Biochem Sci, 19:222-227) may also serve as substrates.

As used herein, the term ligand is synonymous with 25 extracellular signaling molecules, and includes but is not limited to growth factors such as insulin, EGF, PDGF, fibroblast growth factors, vascular endothelial growth factor, and neurotrophins; and cytokines such as growth hormone, erythropoietin, tumor necrosis factor, interleukins 30 and interferons. The term ligand is not limited to soluble molecules, and includes; for example, extracellular matrix proteins, cell adhesion molecules as well as antigenic peptides associated with the major histocompatibility complex proteins on the surface of an antigen-presenting cell.

35 In one embodiment of the invention, the compounds of the invention can be used to trigger or upregulate signal transduction in cells so that the effect of ligand binding to

a receptor is enhanced, or mimicked if the ligand is not present. The compounds exert the effect by inhibiting or diminishing the activity of a phosphatase in the signaling pathway which normally acts negatively toward signaling. One mechanism by which PTPs normally downregulate signal transduction involves the dephosphorylation of specific phosphotyrosine residues (pTyr) on PTKs and their substrates since many PTKs require phosphorylation of some of its own tyrosine residues in order to become optimally active in the signaling pathway. The compounds of the invention can be used to prevent the dephosphorylation of pTyr residues on receptors or their subunits which normally becomes phosphorylated upon ligand binding, thereby enhancing the extent and duration of PTK phosphorylation. The compounds of the invention can also be used to prevent the dephosphorylation of PTKs in which the tyrosine residues become autophosphorylated or transphosphorylated due to its basal activity. In these PTKs, a signal may be triggered by the compounds of the invention in the absence of ligand binding since the basal activity of PTKs is sufficient to promote a signal if constitutive PTP activity is inhibited or diminished by the compounds.

For example, the compounds of the invention may be used to enhance or sustain insulin receptor signal transduction by inhibiting the constitutive dephosphorylation of the pTyr sites on the activated insulin receptor. This would allow the insulin receptor to remain phosphorylated, thus enhancing or sustaining the insulin signal. Furthermore, since it has been shown that insulin receptor is phosphorylated at a low level even in the absence of insulin (Goldstein, 1992, J. Cell Biol., 48:33-42), the compounds of the invention can be used to trigger a signal, even in the absence of insulin, by allowing the tyrosine residues on the receptor to become self-phosphorylated.

Another mechanism by which PTPs may exert a negative effect on signaling is through the dephosphorylation of specific pTyr sites to which SH2-containing molecules bind

during signaling. The absence of such pTyr sites would prevent the recruitment of SH2-containing molecules to specific subcellular compartments to form multiprotein signaling complexes, thereby, preventing the further propagation of the signal. Thus, the compounds of the invention can be used to upregulate or prolong signal transduction by preventing the dephosphorylation of pTyr sites on substrate proteins that normally serve as binding sites for SH2-containing proteins which promote signaling.

In another embodiment of the invention, the compounds of the invention may be used to prevent the dephosphorylation of specific pTyr residues on any substrate, which pTyr residues are essential to the relay or propagation of the signal. Furthermore, the compounds of the invention may be used to prevent the dephosphorylation of specific pTyr residues on any substrate, which pTyr residues are inhibitory to signal transduction.

The compounds of the invention can also be used to suppress or downregulate signal transduction in cells so that the effect of ligand binding to a receptor is abolished or attenuated. The compounds can inhibit a phosphatase in a signaling pathway which normally acts positively toward signaling. For example, PTPs promote signaling through the activation of members of the Src family of PTKs. Src family PTKs have an inhibitory site of phosphorylation in their carboxy termini which by dephosphorylation activates the kinase activity. Thus, the compounds of the invention can be used to prevent the dephosphorylation of the inhibitory pTyr in the carboxy termini of kinases which function normally to promote signal transductions. Src family PTKs may include Src, Fyn, Lck, Lyn, Blk, Hck, Fgr and Yrk. Other kinases which may be similarly regulated by a phosphatase may include Fak and Csk (Taniguchi, 1995, Science 268: 251-255).

The abilities of the compounds of the invention to inhibit protein tyrosine phosphatase activity and to trigger or upregulate a cellular process which is controlled by

signal transduction are demonstrated in the working examples infra.

**5.1. Assays For Determining The Inhibitory  
Activity Of The Compounds**

5 Various procedures known in the art may be used for identifying, evaluating or assaying the inhibition of activity of protein tyrosine phosphatases by the compounds of the invention. In general, such assays involve exposing 10 target cells in culture to the compounds, and a) biochemically analyzing cell lysates to assess the level and/or identity of tyrosine phosphorylated proteins; or (b) scoring phenotypic or functional changes in treated cells as compared to control cells that were not exposed to the test 15 substance.

15 Where mimics of the natural ligand for a signal transducing receptor are to be identified or evaluated, the cells are exposed to the compound of the invention and compared to positive controls which are exposed only to the 20 natural ligand, and to negative controls which were not exposed to either the compound or the natural ligand. For receptors that are known to be phosphorylated at a basal level in the absence of the natural ligand, such as the insulin receptor, the assay may be carried out in the absence 25 of the ligand. Where inhibitors or enhancers of ligand-induced signal transduction are to be identified or evaluated, the cells are exposed to the compound of the invention in the presence of the natural ligand and compared to controls which are not exposed to the compound of the 30 invention.

30 The assays described hereinbelow may be used as a primary screen to evaluate the phosphatase inhibition activity of the compounds of the invention. The assays may also be used to assess the relative potency of a compound by testing a range of concentrations, in a range from 100 $\mu$ M to 1 35 pM, for example, and computing the concentration at which the

amount of phosphorylation or signal transduction is reduced or increased by 50% (IC50) compared to controls.

5.1.1. Biochemical Assays

5 Target cells having a substrate molecule that is phosphorylated or dephosphorylated on a tyrosine residue during signal transduction are exposed to the compounds of the invention and radiolabelled phosphate, and thereafter, lysed to release cellular contents, including the substrate 10 of interest. The substrate may be analyzed by separating the protein components of the cell lysate using a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) technique, in either one or two dimensions, and detecting the presence of phosphorylated proteins by exposing to X-ray 15 film. In a similar technique, without using radioactive labelling, the protein components separated by SDS-PAGE are transferred to a nitrocellulose membrane, the presence of pTyr is detected using an antiphosphotyrosine (anti-pTyr) antibody. Alternatively, it is preferred that the substrate 20 of interest be first isolated by incubating the cell lysate with a substrate-specific anchoring antibody bound to a solid support, and thereafter, washing away non-bound cellular components, and assessing the presence or absence of pTyr on the solid support by an anti-pTyr antibody. This preferred 25 method can readily be performed in a microtitre plate format by an automated robotic system, allowing for testing of large numbers of samples within a reasonably short time frame. Compounds of the present invention were identified and evaluated by this preferred method as described in sections 30 infra.

The anti-pTyr antibody can be detected by labelling it with a radioactive substance which facilitates its detection by autoradiography. Alternatively, the anti-pTyr antibody can be conjugated with an enzyme, such as horseradish 35 peroxidase, and detected by subsequent addition of a calorimetric substrate for the enzyme. A further alternative involves detecting the anti-pTyr antibody by reacting with a

second antibody which recognizes the anti-pTyr antibody, this second antibody being labelled with either a radioactive substance or an enzyme as previously described. Any other methods for the detection of an antibody known in the art may 5 be used.

The above methods may also be used in a cell-free system wherein cell lysate containing the signal-transducing substrate molecule and phosphatase is mixed with a compound of the invention and a kinase. The substrate is 10 phosphorylated by initiating the kinase reaction by the addition of adenosine triphosphate (ATP). To assess the activity of the compound, the reaction mixture may be analyzed by the SDS-PAGE technique or it may be added to substrate-specific anchoring antibody bound to a solid 15 support, and a detection procedure as described above is performed on the separated or captured substrate to assess the presence or absence of pTyr. The results are compared to those obtained with reaction mixtures to which the compound is not added. The cell-free system does not require the 20 natural ligand or knowledge of its identity. For example, Posner et al. (U.S. Patent No. 5,155,031) describes the use of insulin receptor as a substrate and rat adipocytes as target cells to demonstrate the ability of pervanadate to inhibit PTP activity. As another example, Burke et al. 25 (1994, Biochem Biophys Res Comm 204:129-134) describes the use of autophosphorylated insulin receptor and recombinant PTP1B in assessing the inhibitory activity of a phosphotyrosyl mimetic.

In addition to measuring phosphorylation or 30 dephosphorylation of substrate proteins, activation or modulation of second messenger production, changes in cellular ion levels, association, dissociation or translocation of signaling molecules, gene induction or transcription or translation of specific genes may also be 35 monitored. These biochemical assays may be performed using conventional techniques developed for these purposes.

5.1.2. Biological Assays

The ability of the compounds of the invention to modulate the activity of PTPs, which controls signal transduction, may also be measured by scoring for 5 morphological or functional changes associated with ligand binding. Any qualitative or quantitative techniques known in the art may be applied for observing and measuring cellular processes which comes under the control of phosphatases in a signaling pathway. Such cellular processes may include, but 10 are not limited to, anabolic and catabolic processes, cell proliferation, cell differentiation, cell adhesion, cell migration and cell death.

The techniques that have been used for investigating the various biological effects of vanadate as a phosphatase 15 inhibitor may be adapted for use with the compounds of the invention. For example, vanadate has been shown to activate an insulin-sensitive facilitated transport system for glucose and glucose analogs in rat adipocytes (Dubyak et al., 1980, J Biol Chem 256:5306-5312). The activity of the compounds of 20 the invention may be assessed by measuring the increase in the rate of transport of glucose analog, such as 2-deoxy-<sup>3</sup>H-glucose, in rat adipocytes that have been exposed to the compounds. Vanadate also mimic the effect of insulin on glucose oxidation in rat adipocytes (Shechter et al., 1980, 25 Nature 284:556-558). The compounds of the invention may be tested for stimulation of glucose oxidation by measuring the conversion of <sup>14</sup>C-glucose to <sup>14</sup>CO<sub>2</sub>. Moreover, the effect of sodium orthovanadate on erythropoietin-mediated cell 30 proliferation has been measured by cell cycle analysis based on DNA content as estimated by incorporation of tritiated thymidine during DNA synthesis (Spivak et al., 1992, Exp Hematol, 20:500-504). Likewise, the activity of the 35 compounds of the invention toward phosphatases that play a role in cell proliferation may be assessed by cell cycle analysis.

The activity of the compounds of the invention can also be assessed in animals using experimental models of disorders

caused by or related to dysfunctional signal transduction. For example, the activity of the compounds may be tested for its effect on insulin receptor signal transduction in non-obese diabetic mice (Lund et al., 1990, *Nature* 345:727-729), 5 BB Wistar rats and streptozotocin-induced diabetic rats (Solomon et al., 1989, *Am J Med Sci* 297:372-376). The activity of the compounds may also be assessed in animal carcinogenesis experiments since phosphatases can play an important role in dysfunctional signal transduction leading 10 to cellular transformation. For example, okadaic acid, a phosphatase inhibitor, has been shown to promote tumor formation on mouse skin (Suganuma et al., 1988, *Proc Natl Acad Sci* 85:1768-1771).

The data obtained from these cell culture assays and 15 animal studies can be used in formulating a range of dosages for use in humans. The dosage of the compounds of the invention should lie within a range of circulating concentrations with little or no toxicity. The dosage may vary within this range depending on the dosage form employed 20 and the route of administration.

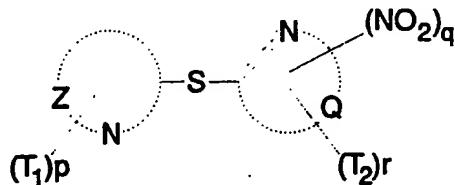
The above-described assays are exemplary and not intended to limit the scope of the invention. Those of skill in the art would appreciate that modifications can be made to the assays to develop equivalent assays that obtain the same 25 result.

#### **5.2. Phosphatase Inhibitors**

The present invention encompasses compounds capable of regulating and/or modulating signal transduction by 30 inhibiting the activity of protein tyrosine phosphatases. More specifically, the present invention encompasses compounds capable of inhibiting protein tyrosine phosphatase activity. These compounds will be referred to herein generically as "phosphatase inhibitors", even though these 35 compounds either upregulate or downregulate cellular processes that are controlled by signal transduction. Generally, the compounds of the present invention may be

heterocyclic nitrogen containing compounds, for example; nitrothiazole compounds or derivatives thereof, as described by the following general formula I:

5



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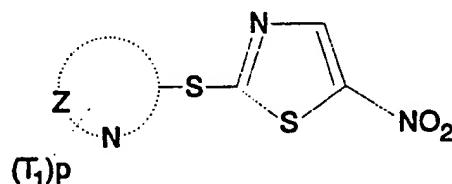
Formula I

wherein:

each of Z and Q, which may be the same or different,  
represents the atoms necessary to complete an unsubstituted  
15 or substituted nitrogen containing heterocyclic ring;  
each of T<sub>1</sub> and T<sub>2</sub> which may be the same or different  
represents alkyl, substituted alkyl, cycloalkyl, substituted  
cycloalkyl, aryl, substituted aryl, aryloxy, halogen, cyano,  
hydroxy, carboxyl, sulfo, carbamoyl, acyl, acylamino,  
20 thioacylamino, sulfamoyl, or sulfonamido; q = 1, 2, or 3, and  
p and r = 0, 1 or 2. In formula I, the nitrogen containing  
heterocyclic nucleus identified by the term "Q" is preferably  
nitropyridine or nitrothiazole. In addition, the present  
invention encompasses pharmaceutically acceptable salts or  
25 analogs of the above compounds.

Preferred compounds of the present invention include  
those of formula II:

30

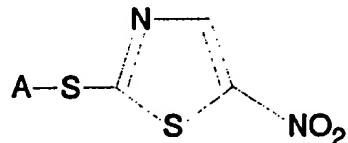


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Formula II

wherein Z, T<sub>1</sub> and p are as defined above, and pharmaceutically acceptable salts thereof.

In another embodiment of the present invention, the compounds of the present invention are described by the  
5 formula III:



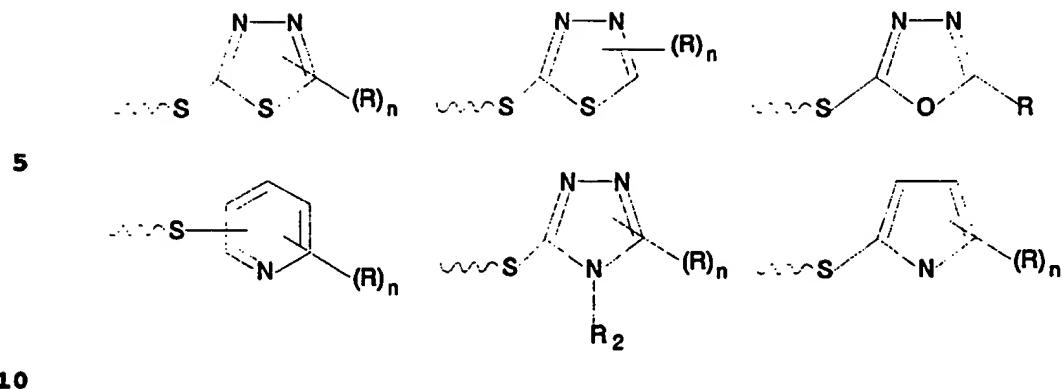
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Formula III

wherein A represents (i) a substituted or unsubstituted monocyclic five or six membered ring having 1-4 hetero ring atoms, at least one of which is nitrogen, the remainder of  
15 which are selected from nitrogen, oxygen or sulfur, e.g., pyridine, pyrrole, imidazole, thiazole, isothiazole, isoxazole, furazan, pyrrolidine, piperidine, imidazolidine, piperazine, oxazole, tetrazole, pyrazole, triazole, oxadiazole, thiadiazole; (ii) a substituted or unsubstituted  
20 monocyclic or fused bicyclic six to ten membered ring having 1 to 4 hetero ring atoms, one of which is nitrogen and the remainder of which are nitrogen, oxygen or sulfur, e.g., indole, quinoxaline, quinoline, isoquinoline, quinazoline, purine; or (iii) a substituted or unsubstituted monocyclic or  
25 fused polycyclic saturated or unsaturated ring having three to 15 atoms, which are carbon, sulfur, nitrogen or oxygen. The invention further encompasses pharmaceutically acceptable salts of the above described compounds. Exemplary structures within group (i) above are:

30

35



wherein:

- 15      R is hydrogen, halogen, cyano, nitro, amino, amido, carboxy, acylamino, hydroxy, alkyl, substituted alkyl, alkoxy, substituted alkoxy, cycloalkyl, substituted cycloalkyl, aryl, substituted aryl, arylalkyl, e.g., benzyl; aryloxy, e.g., phenoxy; a five or six membered heterocyclic 20 ring containing 0 to 3 hetero atoms which are either sulfur, nitrogen or oxygen, which heterocyclic ring may be substituted or unsubstituted;
- 20      R<sub>2</sub> is hydrogen, alkyl, substituted alkyl, alkoxy, substituted alkoxy, cycloalkyl, substituted cycloalkyl, aryl, 25 substituted aryl; arylalkyl, substituted arylalkyl; and n is 0 to 5.

Preferred structures within group (i) above are:



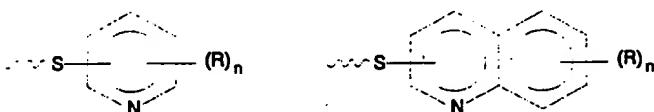
35      wherein:

$R_2$  is hydrogen, alkyl, substituted alkyl, alkoxy, substituted alkoxy, cycloalkyl, substituted cycloalkyl, aryl, substituted aryl; arylalkyl; substituted arylalkyl;

$R_3$  is hydrogen, halogen, cyano, nitro, hydroxy, alkyl, 5 substituted alkyl, alkoxy, substituted alkoxy, cycloalkyl, substituted cycloalkyl, aryl, substituted aryl, arylalkyl; substituted arylalkyl, carboxy, amido, amino, acylamino, sulfonyl, sulfonamido, aminosulfone, a five or six membered heterocyclic ring containing 1 to 2 hetero atoms which are 10 either sulfur, nitrogen or oxygen, which heterocyclic ring may be substituted or unsubstituted.

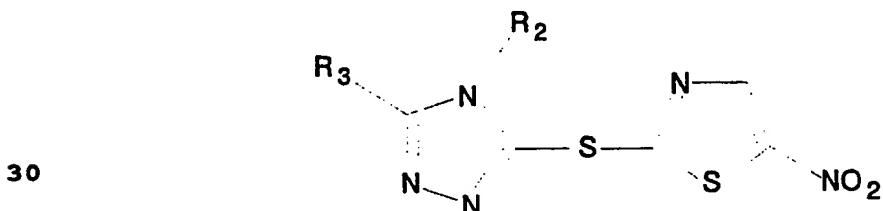
Exemplary structures falling within group (ii) above are:

15



Exemplary structures falling within group (iii) above are 20 cyclopentyl, cyclohexyl, adamantyl, tetrahydroquinoline, tetrahydropyrazole, as well as substituted derivatives thereof.

Specific examples of preferred compounds within the scope of the present invention include but are not limited to 25 compounds of formula (IV):



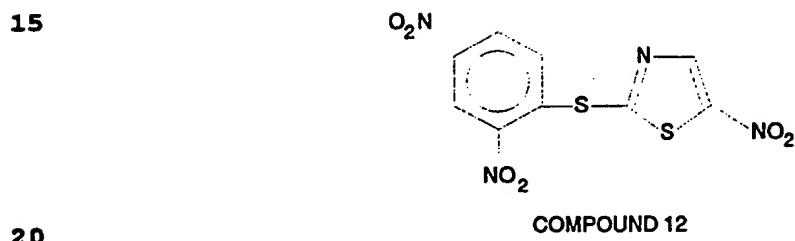
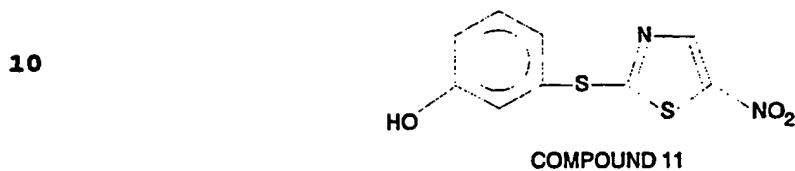
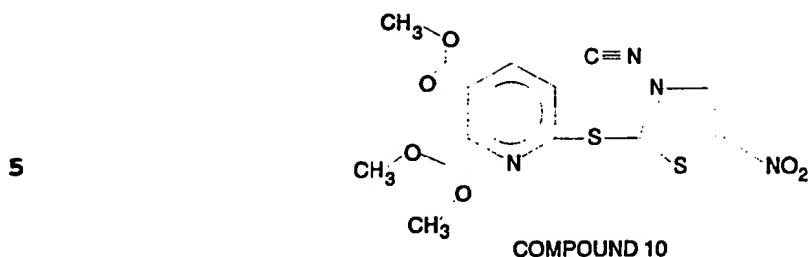
Formula IV

35 wherein R<sub>2</sub> and R<sub>3</sub> are as defined below in Table I.

TABLE I

COMPOUND NO.	R <sub>1</sub>	R <sub>2</sub>	CONCENTRATION	ACTIVITY
5	1	1-ethyl-3-methyl-pyrazole-5-yl	-(CH <sub>2</sub> ) <sub>3</sub> OCH <sub>3</sub>	3.9μM (50%)
	2	t-butyl	H	4.1μM (50%)
	3	thiophene-2-yl	H	13μM (50%)
10	4	OH	cyclohexyl	100μM (30%)
	5	OH	phenyl	13μM (30%)
	6	OH	o-trifluoro-methylphenyl	24μM (30%)
15	7	phenyl	H	53μM (30%)
	8	p-Chlorophenyl	H	100μM (30%)
	9	OH	benzyl	200μM 100μM (35%) (8%)

Generally, the nitrothiazole compounds are electron-accepting compounds, some of which have been reported as light sensitive agents for photographic materials. Compounds 20 within the scope of the present invention are described in U.S. Patent Nos. 5,198,333, 3,870,725 and 3,850,939, and applicants' commonly assigned and copending U.S. patent applications "Pharmaceutical Compositions and Methods for Modulating Signal Transduction" (Pennie & Edmonds Docket No. 25 007683-0123-999, filed June 7, 1996) and "Methods of Inhibiting Phosphatase Activity and Treatment of Disorders Associated Therewith" (Serial No. 08/481,954, Pennie & Edmonds Docket No. 007683-0073-999, filed June 7, 1995), which are incorporated by reference herein in their entirety. 30 Compounds within the scope of the present inventions also include:



and pharmaceutically acceptable salts thereof. In addition, it has been found that the sulfur linkage between the 25 nitrothiazole ring and the adjacent ring can be substituted by an amino linkage, e.g., -NR'- such as in the compound of formula V



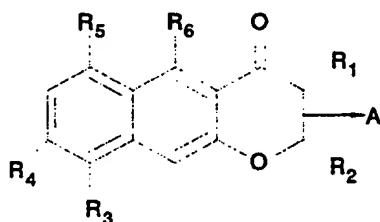
Formula V

35 wherein A is as defined above, and R' is hydrogen, C<sub>1</sub>-C<sub>4</sub> alkyl and substituted C<sub>1</sub>-C<sub>4</sub> alkyl.

Such compounds also possess potent activity in inhibiting or promoting phosphatase activity. Thus, the invention encompasses the above described compounds (see formulas I, II, III and IV) wherein the thio linkage is 5 replaced by an amino linkage.

The compounds of the present invention may also include naphthopyrones and derivatives thereof which is described by the formula (VI):

10



15

Formula VI

or a pharmaceutically acceptable salt thereof, wherein:

A is a single or double bond;

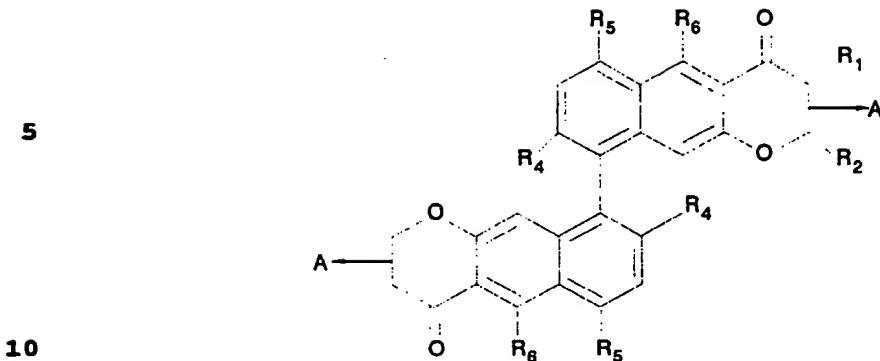
20 R<sub>1</sub>, R<sub>2</sub>, which are the same or different, are C<sub>1</sub> to C<sub>4</sub> alkyl group or an aryl group;

R<sub>4</sub>, R<sub>5</sub>, R<sub>6</sub>, which are the same or different, are H, OH, C<sub>1</sub> to C<sub>4</sub> alkoxy, aryl, C<sub>1</sub> to C<sub>6</sub> alkyl, alkylaryl, arylalkoxy, ester, carboxylic acid, carboxamide, carbamate, or cyano;

25 R<sub>3</sub> is a substituent identical to that of formula VI providing a compound of formula VII:

30

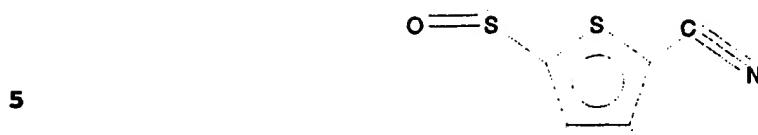
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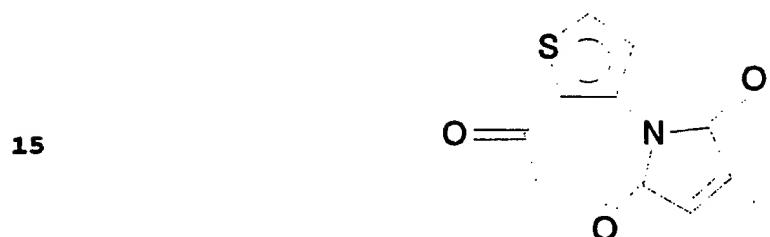
Formula VII

, or a pharmaceutically acceptable salt thereof; or R<sub>3</sub> is an  
 15 aryl group, e.g., mono, bi or tricyclic aryl group; which  
 links to formula VI directly or by amide, carbon or ketone  
 groups.

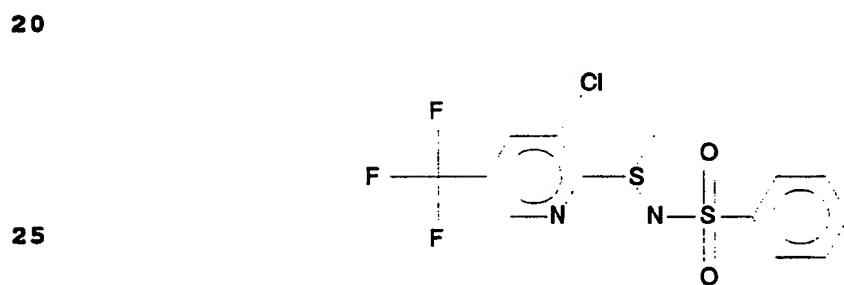
Importantly, other compounds are also included within  
 the scope of the invention. Although somewhat chemically  
 20 dissimilar, on first blush, from the above described series  
 of compounds, the following other compounds are embodied  
 within the invention and demonstrate an ability to regulate  
 and/or modulate signal transduction. In particular, the  
 25 compounds of the present invention, including those shown  
 below, have a unique ability to inhibit phosphatase activity.  
 While not being limited to any theory or explanation, these  
 other compounds are also thought to demonstrate such activity  
 by mimicking the transition state of phosphates departing  
 30 phosphotyrosines. These other compounds include the  
 compounds shown below. Such compounds are available from the  
 Maybridge Chemical Co., Ltd., Trevillet, Tintagel, Cornwall  
 PL34 OHW U.K., and are listed below by their compound number  
 with their Maybridge I.D. number in parentheses.  
 35 Alternatively, these compounds can be synthesized by the  
 methods described herein.



10 COMPOUND 13 (KM 03775)



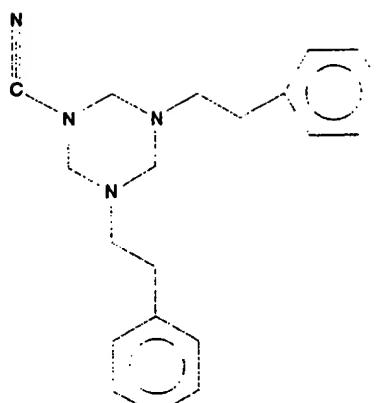
COMPOUND 14 (GK 01415)



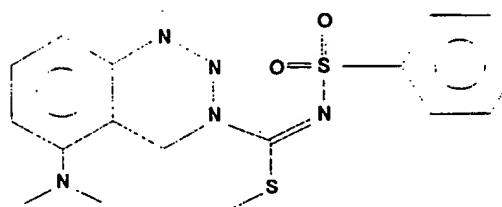
COMPOUND 15 (KM 05704)

30

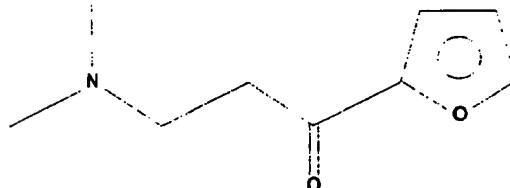
35

**5****10**

COMPOUND 16 (CD 05179)

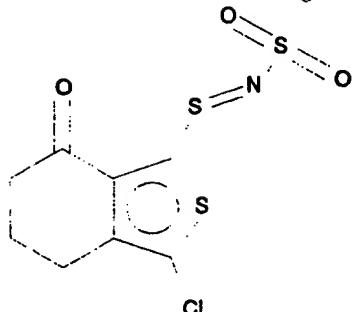
**15****20**

COMPOUND 17 (CD 09806)

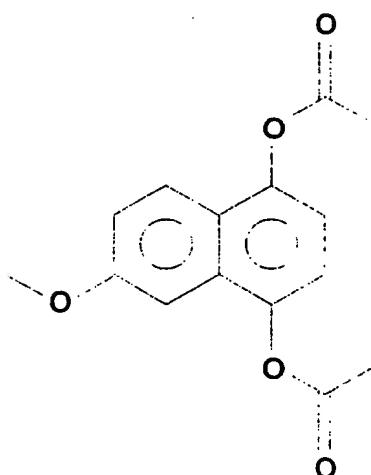
**25****30**

COMPOUND 18 (MWP 01150)

**35**

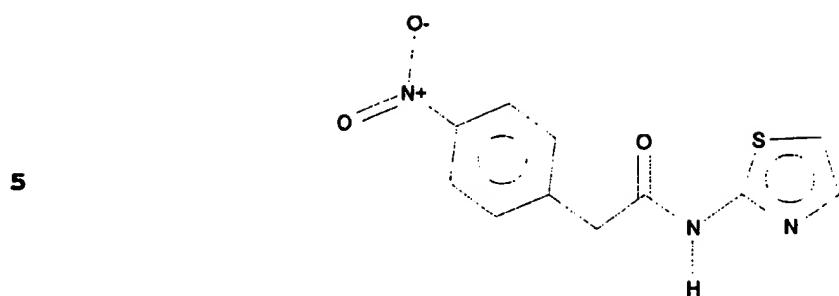
**5****10****15**

COMPOUND 19 (KM 05836)

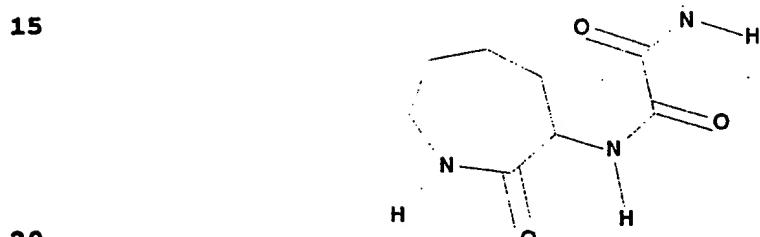
**20****25**

COMPOUND 20 (NRB 01795)

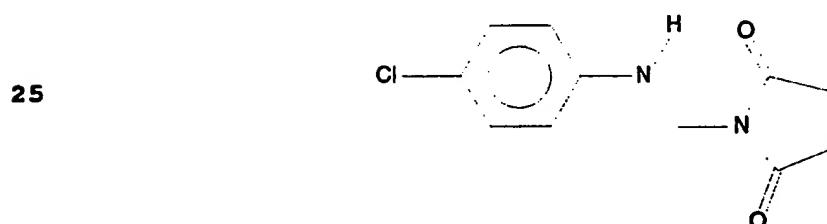
**30****35**



COMPOUND 21 (RF 00491)



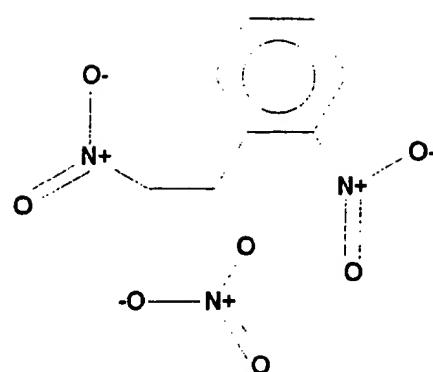
COMPOUND 22 (CD 10481)



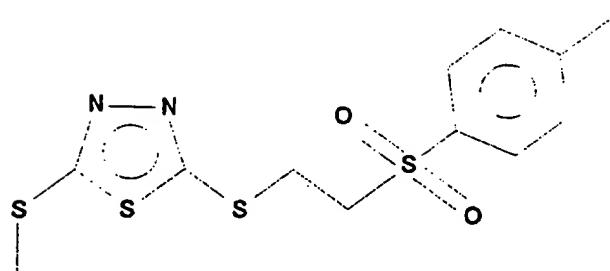
COMPOUND 23 (CD 04397)

30

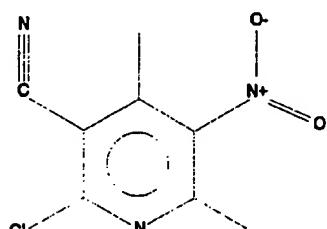
35

**5****10**

COMPOUND 24 (NRB 01455)

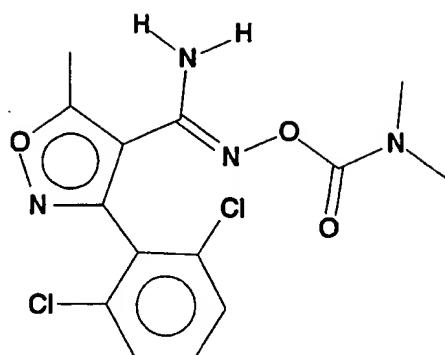
**15****20**

COMPOUND 25 (KM 06413)

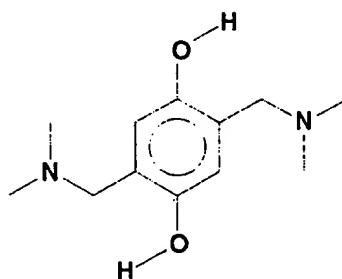
**25**

COMPOUND 26 (RF 00087)

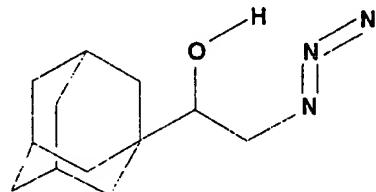
**30****35**

**5****10**

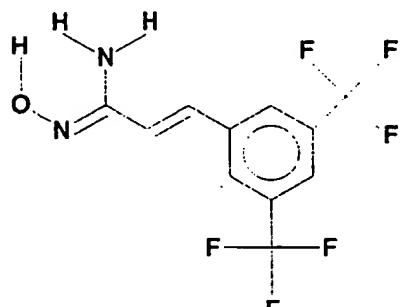
COMPOUND 27 (RF 00807)

**15****20**

COMPOUND 28 (BTB 10421)

**25**

COMPOUND 29 (NRB 02535)

**30****35**

COMPOUND 30 (CD 04728)

The compounds of the present invention can be synthesized in accordance with standard organic chemistry techniques using readily available starting materials.

The present invention is further directed to 5 pharmaceutical compositions comprising a pharmaceutically effective amount of the above-described compounds and a pharmaceutically acceptable carrier or excipient. Such a composition is believed to inhibit the activity of protein tyrosine phosphatases which may be useful in treatment of 10 diseases related to dysfunctional signal transduction, including diabetes and cancer. Alternatively, such composition may act directly on the cells responsible for the disease (e.g., tumor cells). More particularly, the compositions of the present invention may be included in 15 methods for treating, among other diseases, diabetic retinopathy, glioma, melanoma, Kaposi's sarcoma, hemangioma and ovarian, breast, lung, pancreatic, prostate, colon and epidermoid cancer.

Finally, the present invention is also directed to 20 methods for treating diseases, including but not limited to metabolic disorders, diabetes, diabetic retinopathy, rheumatoid arthritis, anemia, immunodeficiency, inflammation, neurodegenerative diseases, and cancer and more particularly cancer related to solid cell tumor growth (e.g., 25 glioblastoma, melanoma and Kaposi's sarcoma and ovarian, lung, mammary, prostate, pancreatic, colon and epidermoid carcinoma).

#### 5.2.1. Analoxes And/Or Salts

30 As used herein, "pharmaceutically acceptable salt" refers to those salts which retain the biological effectiveness and properties of the compound and which are obtained by reaction with inorganic acids or bases such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, methanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid, salicylic acid and the like.

The invention is further directed, where applicable, to solvated as well as unsolvated forms of the compounds (e.g., hydrated forms) having the ability to regulate and/or modulate phosphatase activity.

- 5       The compounds described above may be prepared by any process known to be applicable to the preparation of chemically-related compounds. Suitable processes are illustrated by the representative examples provided, *infra*. Necessary starting materials may be obtained by standard  
10 procedures of organic chemistry.

**5.3. Pharmaceutical Formulations And Routes Of Administration**

The identified compounds can be administered to a human patient, by itself, or in pharmaceutical compositions where  
15 it is mixed with suitable carriers or excipient(s) at doses to treat or ameliorate a variety of disorders, including metabolic disorders, anemia, immunodeficiency, inflammation, neurodegenerative disorders, solid cell tumor growth, including Kaposi's sarcoma, glioblastoma, and melanoma and  
20 ovarian, lung, mammary, prostate, pancreatic, colon and epidermoid carcinoma, diabetes, diabetic retinopathy, hemangioma and rheumatoid arthritis. A therapeutically effective dose further refers to that amount of the compound sufficient to result in amelioration of symptoms of  
25 uncontrolled vasculogenesis and angiogenesis. Techniques for formulation and administration of the compounds of the instant application may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition.

- 30       The formulations of the present invention normally will consist of at least one compound of the invention mixed with a carrier, or diluted by a carrier, or enclosed or encapsulated by an ingestible carrier in the form of a capsule, sachet, cachet, paper or other container or by a  
35 disposable container such as an ampoule. A carrier or diluent may be a solid, semi-solid or liquid material, which

serves as a vehicle, excipient or medium for the active therapeutic substance.

Some examples of the diluents or carriers which may be employed in the pharmaceutical compositions of the present invention are lactose, dextrose, sucrose, sorbitol, mannitol, propylene glycol, liquid paraffin, white soft paraffin, kaolin, microcrystalline cellulose, calcium silicate, silica polyvinylpyrrolidone, cetostearyl alcohol, starch, gum acacia, calcium phosphate, cocoa butter, oil of theobroma, arachis oil, alginates, tragacanth, gelatin, syrup B.P., methyl cellulose, polyoxyethylene sorbitan monolaurate, ethyl lactate and propylhydroxybenzoate, sorbitan trioleate, sorbitan sesquioleate and oleyl alcohol.

15        **5.3.1.      Routes Of Administration**

Suitable routes of administration may, for example, include oral, rectal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as 20 intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections; transdermal, topical, vaginal and the like. Dosage forms include but are not limited to tablets, troches, dispersions, suspensions, suppositories, solutions, capsules, creams, 25 patches, minipumps and the like.

Alternately, one may administer the compound in a local rather than systemic manner, for example, via injection of the compound directly into a solid tumor, often in a depot or sustained release formulation.

30        Furthermore, one may administer the drug in a targeted drug delivery system, for example, in a liposome coated with tumor-specific antibody. The liposomes will be targeted to and taken up selectively by the tumor.

5.3.2. Composition/Formulation

The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, 5 dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers 10 comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the agents of the invention may be 15 formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such 20 penetrants are generally known in the art.

For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be 25 formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Pharmaceutical preparations for oral use can be obtained solid excipient, optionally grinding a resulting mixture, and processing the 30 mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat 35 starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP).

If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For 5 this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be 10 added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, 15 sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft 20 capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

25 For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered 30 in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit 35 may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g., gelatin for use in an inhaler or insufflator may be formulated containing a

powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or 5 continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain 10 formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions 15 of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain 20 substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated 25 solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal 30 compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. 35 Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the

compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble 5 salt.

A pharmaceutical carrier for the hydrophobic compounds is a cosolvent system comprising benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase. The cosolvent system may be the VPD co-solvent 10 system. VPD is a solution of 3% w/v benzyl alcohol, 8% w/v of the nonpolar surfactant polysorbate 80, and 65% w/v polyethylene glycol 300, made up to volume in absolute ethanol. The VPD co-solvent system (VPD:5W) consists of VPD diluted 1:1 with a 5% dextrose in water solution. This co- 15 solvent system dissolves hydrophobic compounds well, and itself produces low toxicity upon systemic administration. Naturally, the proportions of a co-solvent system may be varied considerably without destroying its solubility and toxicity characteristics. Furthermore, the identity of the 20 co-solvent components may be varied: for example, other low-toxicity nonpolar surfactants may be used instead of polysorbate 80; the fraction size of polyethylene glycol may be varied; other biocompatible polymers may replace polyethylene glycol, e.g., polyvinyl pyrrolidone; and other 25 sugars or polysaccharides may substitute for dextrose.

Alternatively, other delivery systems for hydrophobic pharmaceutical compounds may be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Certain organic solvents 30 such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various of sustained- 35 release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for

a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

5       The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such 10 as polyethylene glycols.

In addition to the common dosage forms set out above, the compounds of the present invention may also be administered by controlled release means and/or delivery devices including Alzet® osmotic pumps which are available 15 from Alza Corporation. Suitable delivery devices are described in U.S. Patent Nos. 3,845,770; 3,916,899; 3,536,809; 3,598,123; 3,944,064 and 4,008,719, the disclosures of which are incorporated in their entirety by reference herein.

20       Many of the phosphatase modulating compounds of the invention may be provided as salts with pharmaceutically compatible counterions. Pharmaceutically compatible salts may be formed with many acids, including but not limited to hydrochloric, sulfuric, acetic, lactic, tartaric, malic, 25 succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms.

#### 5.3.3. Effective Dosage

30       Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. More specifically, a therapeutically effective amount means an amount effective to prevent 35 development of or to alleviate the existing symptoms of the subject being treated. Determination of the effective amounts is well within the capability of those skilled in the

art, especially in light of the detailed disclosure provided herein.

For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially 5 from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC<sub>50</sub> as determined in cell culture (i.e., the concentration of the test compound which achieves a half-maximal inhibition of the PTP 10 activity). Such information can be used to more accurately determine useful doses in humans.

A therapeutically effective dose refers to that amount of the compound that results in amelioration of symptoms or a prolongation of survival in a patient. Toxicity and 15 therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD<sub>50</sub> (the dose lethal to 50% of the population) and the ED<sub>50</sub> (the dose therapeutically effective in 50% of the population). The 20 dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio between LD<sub>50</sub> and ED<sub>50</sub>. Compounds which exhibit high therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in 25 formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED<sub>50</sub> with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of 30 administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p1).

35 Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the phosphatase modulating effects, or

minimal effective concentration (MEC). The MEC will vary for each compound but can be estimated from *in vitro* data; e.g., the concentration necessary to achieve a 50-90% inhibition of the phosphatase using the assays described herein. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. However, HPLC assays or bioassays can be used to determine plasma concentrations.

Dosage intervals can also be determined using the MEC value. Compounds should be administered using a regimen which maintains plasma levels above the MEC for 10-90% of the time, preferably between 30-90% and most preferably between 50-90%.

Usual patient dosages for systemic administration range from 1 to 2000 mg/day, commonly from 1 to 250 mg/day, and typically from 10 to 150 mg/day. Stated in terms of patient body weight, usual dosages range from 0.02 to 25 mg/kg/day, commonly from 0.02 to 3 mg/kg/day, typically from 0.2 to 1.5 mg/kg/day. Stated in terms of patient body surface areas, usual dosages range from 0.5 to 1200 mg/m<sup>2</sup>/day, commonly from 0.5 to 150 mg/m<sup>2</sup>/day, typically from 5 to 100 mg/m<sup>2</sup>/day. Usual average plasma levels should be maintained within 50 to 5000 µg/ml, commonly 50 to 1000 µg/ml, and typically 100 to 500 µg/ml.

In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

Desirable blood levels may be maintained by a continuous infusion of the compound as ascertained by plasma levels measured by HPLC. It should be noted that the attending physician would know how to and when to terminate, interrupt or adjust therapy to lower dosage due to toxicity, or bone marrow, liver or kidney dysfunctions. Conversely, the

attending physician would also know to adjust treatment to higher levels if the clinical response is not adequate (precluding toxicity).

The magnitude of a prophylactic or therapeutic dose of 5 the compound in the acute or chronic management of disease will vary with the severity of the condition to be treated and the route of administration. Again, it should be noted that the clinician or physician would know when to interrupt and/or adjust the treatment dose due to toxicity or bone 10 marrow, liver or kidney dysfunctions. The dose, and perhaps the dosage frequency, will also vary according to the age, body weight, and response of the individual patient. In general, as discussed above, the total daily dose ranges for the compounds for the majority of the disorders described 15 herein, is from about 0.02 to about 25 mg/kg patient.

Preferably, a daily dose range should be between about 0.02 to about 3 mg/kg, while most preferably a daily dose range should be between about 0.2 to about 1.5 mg/kg per day. It is further recommended that infants, children, and patients 20 over 65 years, and those with impaired renal, or hepatic function, initially receive low doses, and that they be titrated based on individual clinical response(s) and blood level(s). It may be necessary to use dosages outside these ranges in some cases as will be apparent to those of ordinary 25 skill in the art.

#### 5.3.4. Packaging

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage 30 forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. Compositions comprising a compound of the invention formulated in a compatible 35 pharmaceutical carrier may also be prepared, placed in an appropriate container, and labelled for treatment of an indicated condition. Suitable conditions indicated on the

label may include treatment of a tumor, such as a glioma or glioblastoma and inhibition of angiogenesis.

**5.4. Methods Of Treatment**

5 Any compound of the invention which inhibits or diminishes the PTP activity in a signaling pathway may be used in the therapeutic methods of the invention. In a preferred embodiment, the activity of the compound is sufficiently specific for the PTPs in the pathway so that the 10 compound does not interfere with the function of other phosphatases in the cell. The compounds of the invention may be identified and evaluated by, for example; methods described in section 7 infra.

The compounds and pharmaceutical compositions of the 15 invention can be used for treating diabetes mellitus. The pathogenesis of diabetes generally involves insufficient or a total lack of insulin signal transduction. The paucity or absence of the insulin signal may be caused by a variety of reasons such as a lack of insulin, loss of binding affinity, 20 defective receptor or underexpression of receptor. Insulin receptor activity can be modulated by inhibiting the phosphatases in the signaling using the compounds of the invention. Unlike currently available treatment modalities that are based on the insulin receptor, the insulin signal 25 may be restored or stimulated in cells through the inhibition of dephosphorylating activity, even in the absence of insulin. The example of diabetes mellitus illustrates the principles of therapeutic applications of the invention which may be applied to other disorders that implicate signal 30 transduction by phosphotyrosine phosphatases.

The compounds and pharmaceutical compositions of the invention may be used to treat immune disorders in which cytokine signal transduction is deficient. Cytokines plays a crucial role in hemopoiesis as well as coordinating immune 35 and inflammatory responses. The compounds may be used to replace or enhance the activity of a cytokine in signaling the differentiation and proliferation of hemopoietic cells,

as well as B and T cells in response to antigenic stimulation, and thus be useful for treating anemia and immunodeficiency. The compounds may also be used as an anti-inflammatory agent to treat disorders such as rheumatoid 5 arthritis. The compounds may also be therapeutically useful in treating neurodegenerative diseases by stimulating the growth and differentiation of neuronal cells which is regulated by neurotrophin-mediated signal transduction.

In another embodiment of the invention, the compounds 10 and pharmaceutical compositions of the invention may be used to treat cancer, such as glioma, melanoma, Kaposi's sarcoma, hemangioma and ovarian, breast, lung, pancreatic, liver, prostate, colon and epidermoid cancer, in which the malignant cells proliferate and/or metastasize as a result of 15 uncontrolled signal transduction mediated by growth factors. For example, overexpression of a PTK, such as HER2 has been shown to correlate with the aberrant growth characteristics of tumor cells. Vasculogenesis and/or angiogenesis that facilitates tumor growth may also be inhibited by the 20 compounds. The compounds may modulate signal transduction in these tumor cells so that normal growth characteristics are restored. The compounds may also be useful in treating psoriasis which is caused by excessive epidermal growth factor mediated signal transduction.

25 Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

30

**6. EXAMPLE: DEMONSTRATION OF PHOSPHATASE INHIBITION ACTIVITY OF THE COMPOUNDS**

**6.1. Phosphotyrosine Enzyme Linked Immunosorbent Assay - Insulin Receptor**

35 In this example, the ability of the compounds of the invention to inhibit dephosphorylation of phosphotyrosine (pTyr) residues on insulin receptor (IR) is described. The

assay may be used with any compounds of the invention. Those skilled in the art will recognize that other substrate molecules, such as platelet derived growth factor receptor, may be used in the assay by using a different target cell and anchoring antibody. By using different substrate molecules in the assay, the activities of the compounds of the invention toward different protein tyrosine phosphatases may be assessed. In the case of IR, an endogenous kinase activity is active at low level even in the absence of insulin binding. Thus, no insulin is needed to stimulate phosphorylation of IR. After the exposure to a compound, cell lysates were prepared and added to microtitre plates coated with anti-insulin receptor antibody. The level of phosphorylation of the captured insulin receptor was detected using an anti-pTyr antibody and an enzyme-linked secondary antibody.

#### 6.1.1. Materials And Methods

1. The cell line used for the IR assay was NIH3T3 (ATCC# CRL 1658) engineered to over-express the human IR (H25 cells). Growth media for these cells is DMEM (Gibco) containing 10% fetal bovine serum, 1% L-glutamine, and 20mM Hepes.
2. The anchoring antibody used was BBE which recognizes the extracellular domain of human IR, and was purified by the Enzymology Laboratories, Sugen Inc.
3. PBS (Gibco): KH<sub>2</sub>PO<sub>4</sub> 0.20 g/l, K<sub>2</sub>HPO<sub>4</sub> 2.16 g/l, KCl 0.20 g/l, NaCl 8.00 g/l, pH7.2.
4. Rabbit polyclonal antiphosphotyrosine antibody (anti-pTyr) was prepared by the Enzymology Laboratories, Sugen, Inc.
5. Goat anti-rabbit IgG POD conjugate (Tago, Burlingame, CA, Cat.No. 6430) was used as the secondary antibody.
- 35 6. TBST buffer: 50 mM Tris-HCl, 150 mM NaCl, 0.1% Triton X-100, adjusted to pH7.2 with 10N HCl.

7. Blocking buffer: PBS plus 5% milk (Carnation instant non-fat dry milk).

8. 5X HNTG buffer: 100 mM HEPES, 750 mM NaCl, 50% glycerol, 0.5% Triton X-100, pH 7.5.

5 9. ABTS solution: 100 mM citric acid, 250 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.5 mg/ml ABTS (2,2'-azinobis(3-ethylbenzthiazlinesulfonic acid), adjusted to pH 4.0 with 1N HCl.

10. Cell lysis buffer: HNTG containing 1mM Na<sub>3</sub>VO<sub>4</sub> (0.5M solution kept as a 100X stock at -80°C in aliquots), 10 5mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub> and 5mM EDTA prepared fresh nad keep on ice until ready for use.

11. Hydrogen peroxide: 30% solution.

#### 6.1.2. Preparation Of Assay Plates

15 Microtitre plates (96-well, Easy Wash ELISA plate, Corning 25805-96) were coated with the anchoring antibody at 0.5µg per well, in 100µl PBS for at least two hours at room temperature or overnight at 4°C. Before use, the coating buffer was replaced with 100 µl blocking buffer, and the 20 precoated assay plate was shaken at room temperature for 30 minutes. The wells were washed 3 times with water and once with TBST buffer before adding lysate.

#### 6.1.3. Seeding Cells

25 Cells were grown in 15cm culture dish (Corning 25020-100) in DMEM media containing 10% fetal bovine serum (FBS) until 80-90% confluent. The cells were harvested with trypsin-EDTA (0.25%, 0.5ml, Gibco), resuspended in fresh medium containing 10% FBS, 1% L-glutamine and Hepes, and 30 transferred to round bottom 96-well tissue culture plates (Corning 25806-96) at 25,000 cells/well, 100µl/well. The cells were incubated at 37°C at 5% CO<sub>2</sub> for 24 hours. The media was changed by inverting the plate, and adding DMEM medium containing 0.5% FBS and Hepes. The cells were further 35 incubated overnight at 37°C, 5% CO<sub>2</sub>.

**6.1.4. Assay Procedur**

The assay was set up in the 96-well tissue culture plate. Before adding the compounds to the cells, media in the wells was replaced by serum free DMEM medium, 90 $\mu$ l per well. Positive control wells receive 80 $\mu$ l DMEM. Negative controls received 90 $\mu$ l DMEM. The compounds of the invention were diluted 1:10 with DMEM and 10 $\mu$ l/well of the diluted test substances were transferred to the cells in the wells to achieve a final dilution of 1:100. Positive and negative control wells received 10 $\mu$ l/well of dimethyl sulphoxide (DMSO) to achieve a final concentration of 1%. Positive control wells additionally received 10 $\mu$ l/well of 0.1M Na<sub>3</sub>VO<sub>4</sub> so that the final concentration is 10mM. The tissue culture plate was shaken for 1 minute before incubation at 37°C, 5% CO<sub>2</sub>. After 90 minutes of incubation, the media was removed by inversion of the plate, and 100 $\mu$ l/well of lysis buffer was added to the cells. The tissue culture plate was shaken for 5 minutes and then placed on ice for 10 minutes. The cells were homogenized by repeated aspirating and dispensing, and the lysate was transferred to the corresponding wells of a precoated assay plate.

The substrate in the cell lysates was allowed to bind to the anchoring antibody for 1 hour shaking at room temperature. The lysate was then removed, and the assay plate was washed. All ELISA plate washings were done by rinsing in water 3 times followed by one rinse with TBST. The plate was dried by tapping it on paper towels. Phosphotyrosine was detected by adding 100 $\mu$ l/well anti-pTyr antiserum diluted 1:3000 with TBST to the wells and incubating for 30 minutes shaking at room temperature. The unbound excess anti-pTyr antiserum was then removed, and the assay plate was washed as described above. A secondary antibody diluted 1:3000 with TBST, was added to the wells, and incubated for 30 minutes shaking at room temperature. The secondary antibody was then removed, the plate washed, and fresh ABTS/H<sub>2</sub>O<sub>2</sub> (1.2 $\mu$ l 30% H<sub>2</sub>O<sub>2</sub> to 10ml 0.5mg/ml 2,2'-azinobis(3-ethylbenzethiazline)sulfonic acid in 100mM citric

acid, 250mM Na<sub>2</sub>HPO<sub>4</sub>, pH4.0) was added to start color development. The reaction was stopped after 10 minutes by adding 100μl/well of 0.2M HCl, and incubating and shaking for 1 minute. Absorbance values at 410 nm were measured by a 5 ELISA plate reader (Dynatec MR5000).

#### 6.1.5. Experimental Results

Results of several nitrothiazole compounds of the invention are presented in Table I above. The activity of 10 the compounds are represented by the concentration of the compound which produces the indicated percentage increase in the content of phosphotyrosine over the vanadate control (see Table I).

Once a compound has been shown to be active in the 15 assay, a range of concentrations of the compound were used in kinetic experiments. As shown in Figure 1, compound 10, (2-[(5-nitrothiazol-2-yl)mercapto]-3-cyano-5-acetoxy-6-dimethoxymethyl-pyridine progressively raised the level of pTyr on insulin receptor over a period of 90 minutes. The 20 increase in the pTyr level is dependent on the dose of compound 10 from 15.6μM to 250μM. The kinetics of the inhibition of dephosphorylation by compound 10 at low dosage is similar to that of 10mM vanadate.

The above results demonstrated that the compounds of the 25 invention are capable of increasing the content of phosphotyrosine on insulin receptor.

The assay may also be used for testing compounds of the invention for their ability to inhibit the dephosphorylation of other substrate molecules, such as insulin-like growth 30 factor 1 receptor (IGF-1R) and epidermal growth factor receptor (EGFR). When assaying the effects of the compounds on the dephosphorylation of IGF-1R, NIH3T3/IGF-1R cells expressing IGF-1R starved in serum free media were seeded in the wells of tissue culture plates at a density 20,000 35 cells/well. The wells of ELISA plate were coated with anti-IGF-1R antibodies. For assaying the effects on EGFR, NIH3T3/EGFR cells expressing EGFR grown in media containing

0.5% for 40 hours were seeded in the wells of 96-well tissue culture plates at a density 10,000 cells/well. The wells of ELISA plate were coated with anti-EGFR antibodies.

**5 6.2. Phosphotyrosine Enzyme Linked Immunosorbent or  
Fluorescent Assay - TrkA Receptor**

This example describes the ability of the compounds of the invention to inhibit dephosphorylation of phosphotyrosine (pTyr) residues on an HA-tagged TrkA receptor (TrkA-HA) overexpressed in PC12 cells in an enzyme-linked immunosorbent assay (ELISA) or fluorescent assay format. The assay may be used with any compounds of the invention. Those skilled in the art will recognize that other substrate molecules, such as platelet derived growth factor receptor, may be used in the assay by using a different target cell and anchoring antibody. By using different substrate molecules in the assay, the activities of the compounds of the invention toward different protein tyrosine phosphatases may be assessed.

Overexpression was achieved by using a cell line stably transfected (clone #21) with a construct expressing TrkA-HA under the CMV promoter (vector pcDNA3, Invitrogen) that was infected with an adenovirus expressing HA-TrkA under the SV40 promoter to boost expression.

The following protocol describes the procedures used to grow the cells, infect them with the virus, treat them with drug and perform the ELISA using Europium (Eu) fluorescence for detection.

**30 6.2.1. Reagents And Supplies**

1. Falcon 10-cm tissue culture dishes. (Falcon Cat. # 3003)
2. Falcon 96-well tissue culture plates. (Falcon Cat. # 3072)
- 35 3. Collagen I, bovine. (Collaborative Biomedical, Cat. # 40231)
4. Culture medium for clone #21:

- DMEM (Dulbecco's Modified Eagle Medium).  
(Gibco Cat. # 11965-050)
  - + 10% HS (horse serum), (Gibco Cat # 16050-23)
  - + 5% FBS (fetal bovine serum) (Gibco Cat  
5 # 16000-028)
  - + 1% P/S (Penicillin-Streptomycin) (Gibco Cat  
# 15140-15); and
  - + 400 µg/ml G418 (Geneticin™) (Gibco Cat  
# 11811-031)
- 10 5. PBS Ca<sup>++</sup>- and Mg<sup>++</sup>-free (Dulbecco's Phosphate Buffered Saline). (Gibco Cat # 14190-029)
6. Viral suspension, Ad5SV40-rTrkA-HA. (Neurobiology Lab., Sugen Inc., -20°C. Current stock titer is 4 10<sup>7</sup> pfu (plaque forming units)).
- 15 7. Capture antibody: Anti-HA monoclonal antibody, clone 12CA5 (stock sol. at 0.5 µg/ml). (Boehringer Mannheim Cat. # 1666606)
8. Corning 96-well ELISA plates. (Corning Cat. # 25805-96)
- 20 9. Lysis Buffer, HNTG\* Buffer Formulation:  
20 mM Hepes buffer pH 7.5  
150 mM NaCl  
0.1% Triton X-100  
10% Glycerol
- 25 5 mM EDTA  
1 mM Na<sub>3</sub>VO<sub>4</sub>  
5 mM NaPPi
10. TBST Buffer Formulation:  
50 mM Tris pH 7.2
- 30 150 mM NaCl  
0.1% Tween 20
11. Detection antibody: Biotinylated anti-phosphotyrosine monoclonal antibody, 4G10-Biotin. (Upstate Biotechnology Cat. # 16-103)
- 35 12. Fluorescent probe: Europium-labeled Streptavidin. (Wallac Cat. # 1244-360)

13. Assay buffer, used to dilute Eu-Streptavidin.  
(Wallac Cat. # 1244-106) This can also be home made using  
the following formulation:

1 X TBS  
5           0.005% Tween-40, Sigma Cat. # P-1504  
          0.025% BSA, Sigma Cat. # A-7906  
          0.023% bovine IgG, Sigma Cat. # I-9640  
          10  $\mu$ M DTPA (Diethylenetriaminepentaacetic  
          acid) (Sigma Cat. # D-6518)

10       14. Enhancement solution, used to measure Eu  
fluorescence. (Wallac Cat. # 1244-105)

**6.2.2. Procedure:**

1. Growth of PC12 clone #21.  
15       Clone #21 is a PC12 cell line that expresses TrkA-HA under the CMV promoter (vector pcDNA3, Invitrogen). The cells need to be passed when they are 60-70% confluent (about 1  $10^7$  cells/plate), usually 1/6 once every four days.
2. Viral infection (day 1).  
20       The infection was performed on 40-50% confluent 10-cm plates (3-4 days after splitting) which usually contain 6-8  $10^6$  cells per plate. An MOI (Multiplicity Of Infection) of 3-4, which corresponds to 500  $\mu$ l of the current virus stock at 4  $10^7$  pfu/ml (2  $10^7$  pfu) was used. The viral suspension was added directly to the cells at 5-6 pm so that the virus was in contact with the cells for 16-18 h until they were transferred to 96-well plates the next day.
3. Transfer to 96-well plates (day 2).  
35       - The 96-well plates were precoated with collagen I. The collagen stock was diluted to 10  $\mu$ g/ml with PBS Ca<sup>++</sup>- and Mg<sup>++</sup>-free and add 100  $\mu$ l per well. After several minutes, it was aspirated or shaken out.

- PC12 cells did not need to be trypsinized to be harvested. The plates were simply rinsed with PBS Ca<sup>++</sup>- and Mg<sup>++</sup>-free and resuspend the cells in 10 ml of growth medium (G148 is optional at this stage)
- 5 by pipetting up and down several times. After counting, the cell suspension was adjusted to have 8-10 10<sup>5</sup> cells/ml so that 8-10 10<sup>4</sup> cells were seeded by adding 100 µl per well.
4. Assay (day 3).
- 10 - Corning 96-well ELISA plates were coated with 0.5 µg/well 12CA5 in PBS buffer (100 µl at 5 µg/ml) 2 h at R.T. with agitation.
- In the meantime the cells were treated with drugs. 10 µl of 10 X concentrated drug was added to the 100 µl of medium contained in each well.
- 15 Samples were shaken gently for 1 min. and put back in CO<sub>2</sub> incubator for 2 h.
- After the 2 h coating period with capture antibody, unbound antibody was removed by inverting plate and samples were rinsed once with TBST.
- 20 - After the 2 h incubation with drug, medium was removed by inverting plate, patting on paper towels to absorb excess liquid and adding 100 µl lysis buffer. Samples were shaken vigorously for 2 min. to complete lysis.
- 25 - Lysate was transferred to coated ELISA plate by pipetting up and down a few times and incubating at R.T. for 1 h with agitation.
- Lysate was removed by inverting plates, washing trice with H<sub>2</sub>O, once with TBST and patting on paper towels to absorb excess liquid.
- 30 - 100 µl detection antibody 4G10-Biot was added. At 75 ng/ml in TBST (dilution 1/20,000 of current stock at 1.5 µg/µl). Samples were incubated for 45-60 min. at R.T. with agitation.
- 35 - Detection antibody was removed and washed as above.

- 100  $\mu$ l Eu-Steptavidin at 50 ng/ml in Assay Buffer (dilution 1/2000 of stock sol. at 100 ng/ $\mu$ l) was added. Samples were incubated for 3045 min. at R.T. with agitation.
- 5 - Eu-Steptavidin was removed and washed as above.
- 100  $\mu$ l Enhancement Solution was added and samples were shaken for 5 min. at R.T.
- Reading was done on Wallac DELFIA 1234 fluorometer: Insert plate, press any key to
- 10 activate machine, choose "Auto" then "Group", enter "51" and press "E" for enter.

#### 6.2.3. Results

The results of compounds 13 through 30 are presented in 15 Table II below. The activity of the compounds is represented by the concentration of the compound ( $\mu$ g/ml) which produces a 50% increase in the amount of phosphotyrosine over the control.

TABLE II

Compound No. and Name	Maybridge I.D.	TRK-PTP EC50 ( $\mu$ g/ml)	MW
13 2-cyano-4,4-dimethyl-7-(methylsulfoxo)cyclohexo[c]thiazol-6-one	KM 03775	7.6	267.36
14 2-acetyl-3-maleimidothiazole	GK 01415	26	221.23
15 N-phenylsulfonyl-S-methyl-S-[5-(trifluoromethyl)pyrid-2-yl]sulfoximine	KM 05704	28	382.80
16 5-cyano-1,3-di(2-phenylethyl)hexahydropyrimidine	CD 05179	50	319.45
17 N-[2,6-di(dimethylamino)]-N-phenylsulfonylimino-1-(methylmercapto)methylbenzylamine	CD 09806	73	406.56

	Compound No. and Name	Maybridge I.D.	TRK-PTP EC50 ( $\mu\text{g}/\text{ml}$ )	MW
5	<b>18</b> 2-(3-dimethylamino-1-oxopropyl)furan	MWP 01150	79	167.21
10	<b>19</b> N-(4-chlorophenylsulfonyl)-S-(2-chlorocyclohexo[c]thiazol-6-on-7-yl)-S-methylsulfoximine	KM 05836	97	422.36
15	<b>20</b> 1,4-diacetoxy-6-methoxynaphthalene	NRB 01795	100	274.27
20	<b>21</b> N-(thiazol-2-yl)-4-nitrophenylacetamide	RF 00491	100	263.27
25	<b>22</b> 2-aza-7-[(pyrid-3-yl)methylamino]oxallylamino)cycloheptanone	CD 10481		290.32
30	<b>23</b> N-succinimidomethyl-4-chloroaniline	CD 04397	20% increase at 100 $\mu\text{g}/\text{ml}$	238.67
35	<b>24</b> 1-nitro-2-[2-(nitrophenyl)]-2-nitroxyethane	NRB 01455	20% increase in signal at 12 and 6 $\mu\text{g}/\text{ml}$	257.16
30	<b>25</b> 2-methylmercapto-5-[2-(4-methylphenylsulfonyl)ethylmercapto]-1,3,4-thiadiazole	KM 06413	27% increase at 100 $\mu\text{g}/\text{ml}$	346.49
35	<b>26</b> 2-chloro-3-cyano-4,6-dimethyl-5-nitropyridine	RF 00087	30% increase at 25 $\mu\text{g}/\text{ml}$	211.61
	<b>27</b> 4-[1-amino-1-(dimethylamino)carbonyloxyimino]methyl]-3-(2,6-dichlorophenyl)-5-methylisoxazole	RF 00807	30% increase at 25 $\mu\text{g}/\text{ml}$	357.20

	Compound No. and Name	Maybridge I.D.	TRK-PTP EC50 ( $\mu$ g/ml)	MW
5	<b>28</b> 2,5-di(dimethylaminomethyl)-4-hydroxyphenol	BTB 10421	40% increase at 12 $\mu$ g/ml	224.30
10	<b>29</b> 2-adamantanyl-2-hydroxyethylazide	NRB 02535	41% increase at 100 $\mu$ g/ml	221.30
	<b>30</b> 1-amino-3-[3,5-di(trifluoromethyl)phenyl]-1-(hydroximino)prop-2-ene	CD 04728	55% increase at 12 $\mu$ g/ml	298.19

15 **6.3. Glucose Transport Assay**

This assay was used to assess the ability of the compounds of the invention to inhibit phosphatase activity that is involved in the signaling pathway that regulates the insulin-induced facilitated transport of glucose into adipocytes. It has been shown that incubation of isolated adipocytes with vanadate resulted in a dose-dependent increase in the rate of glucose uptake. Any compounds of the invention may be tested in this assay.

25 **6.3.1. Materials And Methods**

The cell line used for the glucose transport assay was 3T3-L1, a preadipocyte cell line (American Type Culture Collection CL173) which overexpress the insulin receptor. The 3T3-L1 cells were first differentiated by treating cells under confluent growth in DMEM containing 10% fetal bovine serum (FBS) with 0.5mM 3-isobutyl-1-methyl-xanthine, 5  $\mu$ g/ml porcine insulin, 250mM dexamethasone for 2 days. The cells were then grown in DMEM containing 10% FBS and 5  $\mu$ g/ml porcine insulin for two days, after which the cells were cultured in DMEM containing only 10% FBS.

Cells for use in the assay were first grown overnight in DMEM media and 1% FBS at 37°C at 5% CO<sub>2</sub>. Two hours before

use, the overnight media was replaced with serum free DMEM containing 5mM glucose. After washing the cells twice with phosphate buffered saline (PBS), serial dilutions of the compounds of the invention diluted 1:100 into DMEM were added 5 to the wells for a final concentration range of 0.1  $\mu$ M to 500  $\mu$ M. Negative control wells received DMEM only. The cells were incubated with the test compound for 1-4 hours at 37°C at 5% CO<sub>2</sub>. Fifteen minutes before the end of each time point, 2-deoxy-<sup>3</sup>H-glucose was added to a final concentration of 50 $\mu$ M 10 and 0.5 $\mu$ Ci/ml. At the end, the compound was removed, and the wells were washed twice with PBS containing 10mM glucose. The cells were lysed with 50 $\mu$ l 0.5N NaOH, and the cell lysates were transferred to a scintillation vial and mixed 15 with 5.2  $\mu$ l of glacial acetic acid. The wells were washed each with 200  $\mu$ l PBS which was transferred to the corresponding scintillation vial. <sup>3</sup>H radioactivity was counted with a Beckman counter.

#### 6.3.2. Experimental Results

20 The naphthopyrone compounds tested in this assay were able to increase glucose uptake in these cells in the absence of insulin, for example; 2-methyl-5,6,8-trihydroxy-9-(5,6,8-trihydroxy-2-methyl-4H-naphtho[2,3,b]pyrano-4-one-9-yl)4H-naphtho[2,3,b]pyran-4-one (see Figure 2).

25 These data indicate that the compounds of the invention can mimic the effect of insulin in increasing the rate of glucose uptake in adipocytes in the absence of insulin.

It may be apparent to those skilled in the art that modifications and variations of the present invention are 30 possible in light of the above disclosure. It is understood that such modifications are within the spirit and scope of the invention, which is limited and defined only by the appended claims.

What is claimed is:

1. A phosphatase inhibitor that mimics the transition state of a phosphate leaving a tyrosine residue, which comprises a compound having:

5 (i) an aryl group that mimics the structure of tyrosine, and

(ii) a highly diffuse negatively charged moiety that binds a divalent metal ion

so that the compound inhibits the dephosphorylation of  
10 phosphotyrosine residues of a cellular substrate involved in signal transduction.

2. The compound of Claim 1 in which the negative charge generated by the moiety of (b) is diffused into the  
15 aryl moiety.

3. The compound of Claim 1 in which the divalent metal ion is Mg<sup>++</sup>, Mn<sup>++</sup> or Zn<sup>++</sup>.

20 4. The compound of Claim 1 in which the diffuse negatively charged moiety has a hydroxy-ketone functional group.

5. The compound of Claim 1 in which the diffuse  
25 negatively charged moiety has an acidic group.

6. The compound of Claim 1 which stimulates receptor-mediated signal-transduction in the absence of the ligand binding to the receptor.

30

7. The compound of Claim 1 which inhibits receptor-mediated signal-transduction in the absence of the ligand binding to the receptor.

35

1/2

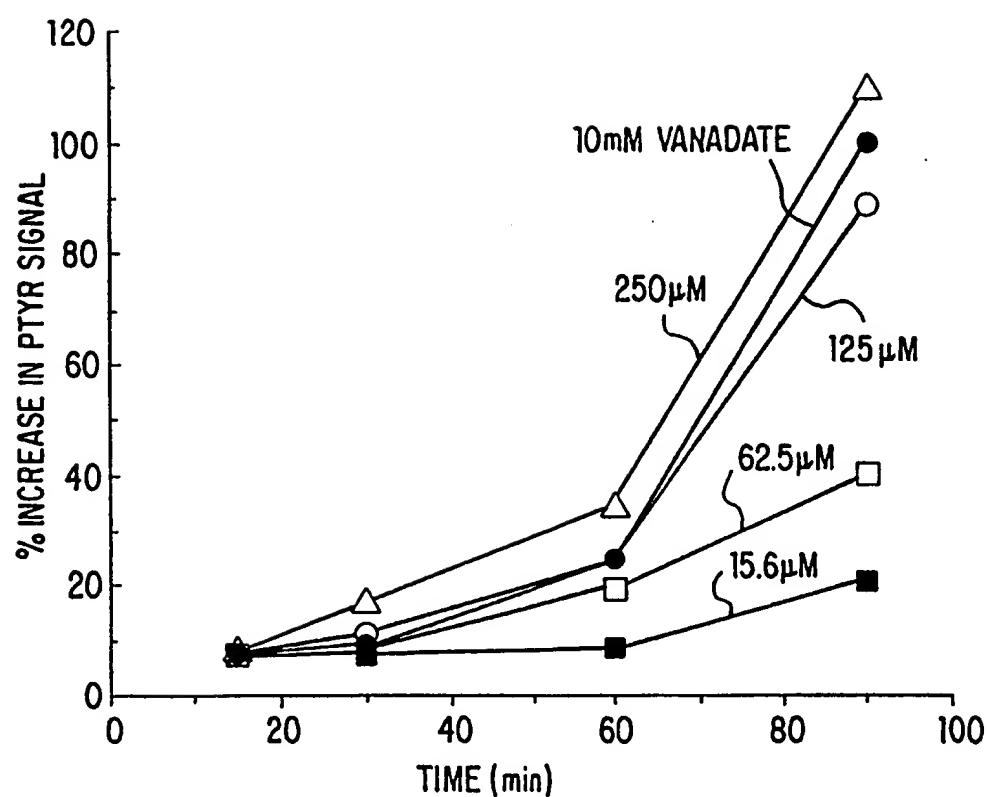


FIG. 1

2/2

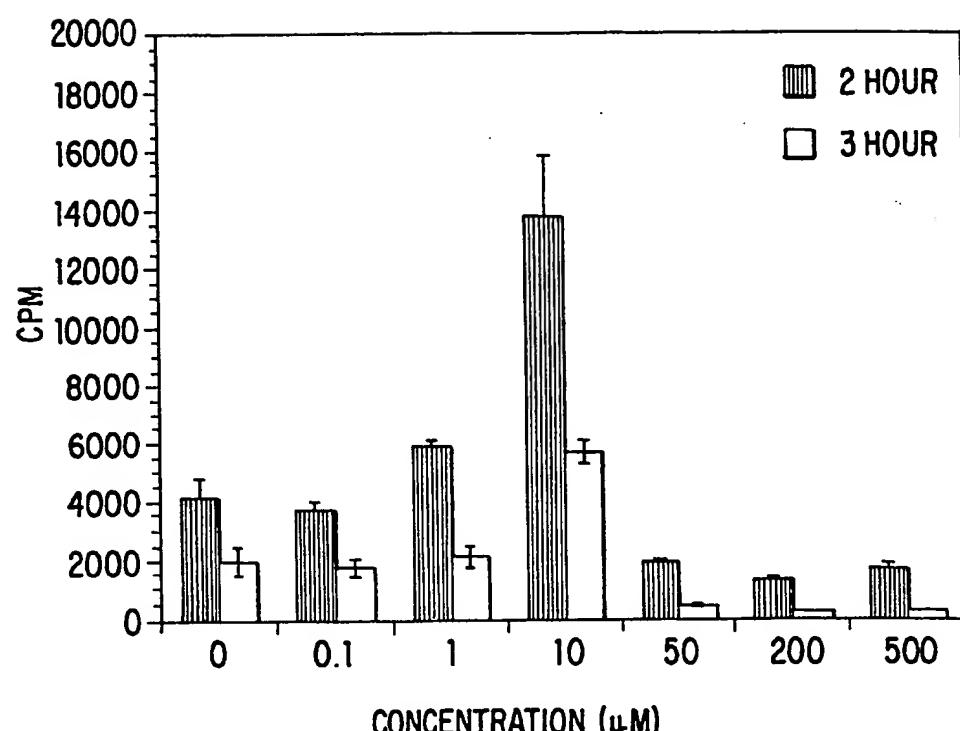


FIG.2